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(54) Title: DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE

(57) Abstract

The invention provides dendritic-like cell/tumor cell hybridomas and pluralities of dendritic-like cell/tumor cell hybrids that confer tumor resistance *in vivo*. The hybrids and hybridomas are generated by the fusion of tumor cells with dendritic-like cells. For instance, immortal tumor cells from an autologous tumor cell line can be fused with autologous or HLA-matched allogeneic dendritic-like cells. Autologous tumor cell lines can be derived from primary tumors and from their metastases. Alternatively, immortal dendritic-like cells from an autologous or allogeneic HLA-matched dendritic-like cell line can be fused with autologous tumor cells. Autologous dendritic-like cell lines can be prepared from various sources such as peripheral blood and bone marrow. Dendritic-like cell/tumor cell hybridomas and pluralities of hybrids can be directly infused for active immunization of cancer patients against their residual tumor cells. The hybridomas and hybrids can also be used for the *in vitro* activation of autologous immune cells before their reinfusion into the patient for passive immunization against the tumor cells.

DENDRITIC-LIKE CELL/TUMOR CELL HYBRIDS AND HYBRIDOMAS FOR INDUCING AN ANTI-TUMOR RESPONSE

Technical Field

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This invention is in the field of immunotherapy for the treatment of cancer. Specifically, the invention provides a hybridoma consisting of a fused tumor cell and a dendritic-like cell, which hybridoma is capable of inducing an anti-tumor response in vivo when administered to a subject in need of anti-tumor treatment.

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Background of the Invention

The Immune Response

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The introduction of pathogens such as bacteria, parasites or viruses into a mammal elicits a response contributing to the specific elimination of the foreign organism. Foreign material is referred to as antigen, and the specific response is called the immune response. The immune response starts with the recognition of the antigen by a lymphocyte, proceeds with the elaboration of specific cellular and humoral

20 effectors and ends with the elimination of the antigen by the specific effectors. The specific effectors are essentially T lymphocytes and antibodies, mediating cellular and humoral immune responses, respectively. The present invention relates to the initiation of a cellular immune response. The initiation of a cellular immune response starts with the recognition of an antigen on the surface of an antigen-presenting cell (APC).

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Antigen Recognition by T-Lymphocytes

Cellular antigen recognition is operated by a subset of lymphocytes called T-lymphocytes. T-lymphocytes include two major functional subsets. They are T-helper

30 lymphocytes (TH), that usually express the CD4 surface marker, and cytotoxic T-lymphocytes (CTL), that usually express the CD8 surface marker. Both T-cell subsets express an antigen receptor that can recognize a given peptide antigen. The peptide needs to be associated with a major histocompatibility molecule (MHC) expressed on the surface of the APC, a phenomenon known as MHC restriction. T-cells bearing the

35 CD4 surface marker recognize peptides associated with MHC class II molecules,

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whereas T-cells bearing the CD8 surface marker recognize peptides associated with MHC class I molecules.

Since the T-cell antigen receptor can only recognize peptides associated with MHC molecules at the surface of an APC, cellular proteins need to be processed into such peptides and transported with MHC molecules to the cell surface. This is referred to as antigen processing. Exogenous proteins, phagocytosed by the APC, are broken down into peptides that are transported on MHC class II molecules to the cell surface, where they can be recognized by $CD4^+$ T-cells. In contrast, endogenous proteins, synthesized by the APC, are also broken down into peptides, but the latter are transported on MHC class I molecules to the cell surface, where they can be recognized by $CD8^+$ T-cells.

When a T-cell binds through its antigen receptor to its cognate peptide-MHC complex on an APC, the binding generates a first signal from the T-cell membrane towards its nucleus. However, this first signal is insufficient to activate the T-cell, at least as measured by the induction of IL-2 synthesis and secretion. Activation only occurs if a second signal or costimulatory signal is generated by the binding of other APC surface molecules to their appropriate receptors on the T-cell surface. The best known costimulatory molecules identified to date on APC are B7-1 (Razi-Wolf et al, 1993, Proc. Natl. Acad. Sci. USA 90: 11182-1186) and B7-2 (Hathcock et al, 1993, Science 262: 905-907); both bind to the CD28/CTLA4 counter-receptor on T-lymphocytes. The capacity to present peptide antigens together with costimulatory molecules in such a way as to activate T-cells is hereafter referred to as antigen presentation. Only APCs have the capacity to present antigens to $CD4^+$ (predominantly TH) and $CD8^+$ (predominantly CTL) T-cells, leading to the development of humoral and cellular immune responses.

T-lymphocyte Activation by Antigen-Presenting Cells

APCs are heterogeneous in their cell lineage and functional performance. They include distinct cell types such as B lymphocytes, T lymphocytes, monocytes/

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macrophages and dendritic cells from myeloid origin. All these cells are bone marrow-derived cells, that need to mature and to be activated in order to function efficiently as APCs.

The functional performances of APCs rely critically upon the nature and state of maturation of the cells included in purified or enriched APC preparations. The latter vary with the tissue of origin and method of purification. In an operational way, we call dendritic-like cells (DLCs)) all non-B cells present in purified or enriched preparations of dendritic cells. These cells all share some morphological, physical or biochemical characteristics with dendritic cells, leading to their co-purification with dendritic cells. Therefore, the term DLCs refers hereafter not only to dendritic cells of myeloid origin, but also to monocytes, T-lymphocytes and other non-Bcells present in enriched or purified dendritic cell preparations. In mice, the spleen is very often used as a source of DLCs (reviewed by Steinman, 1991, Annu. Rev Immunol 9: 271-296). However, mouse DLCs have also been generated by in vitro culture from bone marrow progenitors in the presence of cytokines (Inaba et al, 1992, J. Exp. Med. 176: 1693-1702). In humans, blood or bone marrow are the usual sources of DLCs, that are used either immediately or more often after culture in the presence of cytokines. Several protocols of purification and in vitro culture have been published (reviewed in Young and Inaba, 1996, J. Exp. Med 183: 7-11), and patent applications have been filed for some of them (WO 93/20185, by Steinman R., Inaba K. Schuler G WO 93/20186, by Banchereau J and Caux C; WO 94/02156 by Engleman E.., Markowicz S and Metha A; WO 95/28479 by Brugger W and colleagues of Mertelsmann R.).

T-Lymphocyte Activation by Tumor Cells

There is increasing evidence that tumor cells do not usually function as APCs (reviewed by Young and Inaba, 1996, J. Exp. Med 183: 7-11). Although some tumor cells are capable of delivering an antigen-specific signal to T cells, they may not provide the costimulatory signals which are necessary for the full activation of T-cells and thereby fail to induce an efficient anti-tumor immune response. In order to compensate for this inefficient induction of an anti-tumor immune response, different

approaches have been tried in experimental animals (reviewed by Grabbe et al, 1995, Immunology Today 16: 117-121).

In one such approach, tumor cells were genetically engineered to express one or more molecules known to be involved in antigen presentation on APC. To date, efficient _in vivo_ results from this approach were obtained with tumor cells co-expressing MHC class I, MHC class II and B7-1 molecules, suggesting that the successful immunotherapy was linked to the activation of both CD4^{+} and CD8^{+} T cells. For example, Basker et al, 1995, J. Exp. Med 181:619-629 engineered mouse fibrosarcoma cells, that naturally express MHC class I molecules, to express in addition MHC class II and B7-1 molecules; the injection of these modified tumor cells was sufficient to cure syngeneic mice carrying large established tumors. It should be noted that tumor cells expressing MHC class I but not MHC class II molecules and transduced with the B7-1 costimulator also induced an _in vivo_ anti-tumor immune response, and that the latter depended upon the activation of CD8^{+}, but not CD4^{+} T cells (Ramarathinam et al, 1994, J. Exp. Med. 179: 1205-1214). The disadvantage of this approach lies in the genetic engineering of the tumor cells, a technique that usually involves the use of viral vectors for efficient gene transfer. Viral vectors are not totally safe for the treatment of human patients. The main reason is that they can recombine both _in vitro_ and _in vivo_, which may lead to the production of novel wild type viruses of unpredictable pathogenicity. This limitation stimulated the development of alternative methods of efficient gene transfer, such as the one recently described by Birnstiel and colleagues (WO 94/21808).

In another approach, APCs were loaded with a source of tumor antigens. Amongst the APC tested for such a purpose, DLCs appeared to be the most efficient. To date, it is clear that DLCs pulsed with tumor cell lysates (Knight et al, 1985, Proc. Natl. Acad. Sci. USA 82: 4495-4497), with a purified tumor-associated protein (Flamand et al, 1994, Eur. J. Immunol 24: 605 - 610; Paglia et al, 1996, J. Exp. Med. 183: 317-322) or with tumor-associated peptides (Ossevoort et al, 1995, J. Immunotherapy 18: 86-94; Mayordomo et al, 1995, Nature Medicine, 1: 1297-1302)

can efficiently induce an anti-tumor immune response <u>in vivo</u> There are, however, disadvantages to this approach. Tumor cell lysates or fractions thereof are relatively easy to prepare, but the loading of DLCs with such crude preparations could, at least theoretically, induce adverse auto-immune reactions in the host. Similar secondary

5   effects could be induced by DLCs loaded with all the peptides eluted from tumor cells, as described by Zitvogel et al, J. Exp. Med. 1996, 183: 87-97. The latter risk is reduced by pulsing DLCs with purified, tumor-specific antigens or peptides. However, there are very few known tumor-specific antigens, and in addition, their production and purification are both labor-intensive and expensive.

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In a recent approach, a tumor cell and one sort of APC, namely a B-lymphocyte, were united into a single cell by somatic cell fusion (Guo et al, 1994, Science 263: 518-520). Guo et al. fused a rat hepatoma cell line with <u>in vivo</u>-activated B lymphocytes, and showed that some of the resulting B cell/tumor cell hybridomas

15   induced tumor-resistance in syngeneic rats and also cured the animals of a small pre-established tumor. The selected hybridomas expressed MHC class II restriction elements and B7 costimulatory molecules, which strongly suggested that the immunotherapy worked through the activation of CD4$^+$ TH cells. When compared to the two previous approaches, this third approach has the general advantages of somatic

20   cell fusion, namely, it brings together not only the known tumor antigens and known costimulators of activated B-cells, but possibly some as yet unknown molecules carrying out these functions. When compared to the genetic engineering of tumor cells, this cellular engineering does not require the identification of the genes encoding costimulatory molecules, nor their transfer into tumor cells. Similarly, when compared

25   to the pulsing of APC with purified tumor-specific antigens, somatic cell fusion does not require the identification of genes encoding tumor-specific antigens, nor the production and purification of the corresponding recombinant proteins. However, in its present description, this approach is inapplicable to human cancer patients, because it involves the use of <u>in vivo</u>-activated B-cells as fusion partners of the tumor cells. <u>In</u>

30   <u>vivo</u>-activated B-cells were recovered from the spleen fourteen days after immunization

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with soluble antigen in complete Freund's adjuvant, which cannot be used in humans. In addition, if immunizations are done without Freund's adjuvant, the outcome of an _in vivo_ activation of B-cells remains unpredictable in individual animals, and it is expected to be unpredictable in individual human patients. Finally, the selection of the

5   hybridomas is quite labor-intensive. It required the preparation, absorption and characterization of tumor-specific polyclonal antisera, that were used to select the cells expressing surface markers of the tumor parent; this first selection was then followed by a second selection of cells expressing surface markers of the _in vivo_-activated B-cell parent.

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What is really needed is a method to harness the ability of DLCs to elicit an anti-tumor response, so that the immune system of a subject can mount a rejection of the tumor cells. In addition, this method should be transposable to human cancer patients.

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Summary of the Invention

The present invention provides DLC/tumor cell hybridomas and a plurality of DLC/tumor cell hybrids for use in the treatment of cancers. The hybridomas and

20   hybrids of the invention are capable of inducing an anti-tumor response when administered to the subject, _in vivo_.

A DLC/tumor cell hybridoma of the invention is produced by first providing a sample of the specific tumor against which an immune response is needed. In one

25   embodiment of the invention, an immortal cell line is derived from the tumor sample, and then the tumor cells are fused with DLCs. Preferably, autologous DLCs from the subject are used, but matched HLA-compatible DLCs may also be used as fusion partners. Once the DLCs are fused with the tumor cells, selection is carried out. In this embodiment, hybridomas which exhibit DLCs characteristics are selected, their

30   immortality being necessarily contributed by fusion with the tumor cell. In a second embodiment of the invention, an established immortal human tumor cell line is

provided which expresses at least one of the tumor-associated antigens of the patient's tumor cells.   Cells from the tumor cell line are fused with autologous or HLA-compatible allogeneic DLCs to form hybridomas which are then selected for retention of DLC characteristics. In a third embodiment of the invention, an immortal DLC line

5   is established, and then DLCs of this line are fused with the patient's tumor cells from primary culture.   The resulting hybridomas are selected for retention of DLC characteristics as well as expression of at least one tumor-associated antigen of the patient's tumor cells.

10   In other embodiments of the invention, tumor cells are fused with DLCs, and the resulting plurality of hybrids is used directly for treatment, without selection.

The DLC/tumor cell hybridoma, or plurality of hybrids, is administered to the subject to induce an immune response against residual tumor cells in the subject's

15   circulation or organs.   Alternatively, the hybridoma or plurality of hybrids is co-cultivated in vitro with immune cells from the subject in order to activate against the tumor cell; the activated immune cells are then returned to the subject.

Brief Description of the Drawings

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Figure 1. Scanning electron microscopy of parent cells and of two murine DLC/tumor cell hybridomas (x 4,000). The figure illustrates the "tumor-like"and "dendritic-like"characteristics of two DLC/tumor cell hybridomas. Hybridoma HY1 (Fig. 1c)

25   resembles more the parent P815* tumor cell (Fig. 1a) than the parent dendritic cell (Fig. 1b), whereas hybridoma HY41 (Fig. 1d) resembles more the dendritic cell (Fig. 1b) than the P815* tumor cell (Fig. 1a). It is the "dendritic-like" hybridoma HY41 that was selected for in vivo experiments.

30   Figure 2a-e. FACS analysis of DLC/tumor cell hybridomas HY41 and HY62, showing the expression of CD3 and the TCR V-β8 domain by the CD3-positive subclones

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(HY41 CD3+ and HY62 CD3+); the CD3-negative subclones of these hybridomas (HY41 CD3- and HY62 CD3-) as well as the parent P815* tumor cells fail to express the TCR V-β8 domain.

5   Figure 3. Ethidium bromide-stained gel electrophoresis of Polymerase Chain Reaction products obtained with mouse genomic DNA, using TCR V-β8 and Cβ primers. A rearranged TCRβ gene fragment was amplified from genomic DNA of a mouse T-cell hybridoma (T), as well as from the HY41 (41) and HY62 (62) DLC/tumor cell hybridomas; no rearranged TCRβ fragment was amplified from DNA of P815* tumor

10   cells (P) and spleen cells (S), used as negative controls.

Figure 4. Survival curves of immunocompetent and immunocompromised (i.e. irradiated) DBA/2 mice after ip inoculation with 5 x 10^5 syngeneic hybridoma cells HY41 or with the same number of parental P815* tumor cells.

15   Key: O P815 in normal mice (n = 10)

● P815 in irradiated mice (n = 10)

Δ HY 41 in normal mice (n = 12)

▲ HY 41 in irradiated mice (n = 10)

20   This figure shows that the "dendritic-like" hybridoma HY41 was rejected by 75% (9/12) of the immunocompetent mice, while the parent tumor was rejected, in this particular experiment, by 20% (2/10) of the animals. This difference in survival was not due to a difference in tumorigenicity, since both cell lines killed all (10/10) immunocompromised animals within four weeks of inoculation.

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Figure 5. Survival curves of naive and HY41-treated DBA/2 mice after ip inoculation with 5 x 10^5 syngeneic P815* tumor cells. Y-axis = survival (%). X-axis = weeks after inoculation.

Key: O normal mice (n = 9)

30   ∇ "HY41" - treated mice (n = 9)

This figure shows that the nine HY41-survivors (see Fig. 4) became at least partially resistant to a lethal challenge with the parental P815* tumor cells, and that 4/9 of these animals showed complete tumor resistance for at least three months.

5   Figure 6. P 815 Targets (T). Chromium release assay on P815* and L1210 target cells with spleen cells from individual mice. Y-axis = Cr release (%). X-axis = spleen from individual mice:effectors (E). This figure shows that the spleen cells of the four P815*-resistant mice (see Fig. 5; individual mice nrs 5 - 8 in Fig. 6), contain a strong cytolytic activity directed against P815* cells (Fig. 6A) but not against the irrelevant

10   (but MHC class I-matched) L1210 tumor cells (Fig. 6B). In contrast, the spleen cells of the four naive animals (mice nrs 1-4) do not show any detectable cytolytic activity against P815* cells (Fig. 6A). The spleen cells from individual mice (1 - 8) were cultured in vitro for five days either in the absence (x) or in the presence of P815* stimulator cells (x + P815). Thereafter, they were used as effector cells on chromium-

15   labelled target cells, at different effector:target (E:T) ratios.

Figure 7. Survival of mice bearing an established tumor P815*. Y-axis = % of survival. X-axis = weeks after inoculation.

Key: O   untreated mice (n = 10)

20   ●   HY41 - treated mice (n = 10)

         ∇   HY62 - treated mice (n = 10)

         ▼   P815 - treated mice (n = 10)

Survival curves of tumor-inoculated mice treated with irradiated HY41 or HY62

25   hybridoma cells. All mice were inoculated ip with 2 x 10^5 P815* tumor cells on day 0. The figure shows that 2 months after tumor inoculation, 6/10 and 4/10 animals treated by 4 weekly ip injections of irradiated HY41 and HY62 hybridoma cells, respectively, were alive and tumor-free. In contrast, none (0/10) of the untreated animals and only 2/10 animals treated with irradiated P815* tumor cells were alive at that same time.

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Figure 8. FACS analysis showing HLA-DR expression in human F3BG10 DLC/tumor cell hybridoma (Fig 8a) and in its subclone F3BG10-H12 (Fig 8b) before and after incubation with interferon γ. Before incubation with the cytokine, labeling by the anti-HLA-DR mAb (thinner line) was identical to the labeling by the isotope-matched control mAb (not shown). After 24 hours incubation with interferon γ, around 40% of the F3GG10 hybridoma cells and over 90% of the H12 subclone cells were specifically labeled by the anti-HLA-DR mAb (thicker line).

Detailed Description of the Invention

The present invention provides DLC/tumor cell hybrids and hybridomas for activating anti-tumor responses. Although the specific procedures and methods described herein are first exemplified using a DBA/2 mouse mastocytoma cell line and DLCs isolated from syngeneic spleen, they are merely illustrative for the practice of the invention. Analogous procedures and techniques are applicable for the treatment of human subjects, as thereafter exemplified using a human osteosarcoma cell line and blood-derived DLCs. Therefore, DLC/tumor cell hybrids and hybridomas could be used to immunize human patients against their cancer. Procedures applicable to the treatment of a human subject would involve the following steps.

(1) A sample is provided of the tumor against which an immune response is needed. Such a sample can be obtained when the primary tumor and/or its metastases are removed by surgery, as practised for example for cancers of the breast, prostate, colon, and skin. When the treatment of the cancer involves chemotherapy and/or radiotherapy rather than surgery, as practised for example for small cell lung cancer, lymphomas and leukemias, a sample of the tumor can be obtained from a metastatic site, either before treatment or after relapse. Examples of easily-accessible tumor sampling sites are the peripheral blood, bone marrow, peritoneal and pleural effusions, lymph nodes and skin.

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Tumor cells can be separated from blood or bone marrow samples, for instance, by a combination of physical, enzymatic and immunological methods. Contaminating red blood cells can be removed by osmotic lysis. Tumor cells can be concentrated by density centrifugation. Tumor cells can be separated from other cells

5   by binding antigen on the tumor cell surface to antibody-coupled magnetic beads, which are then separated from the biological fluid by means of magnets. In negative cell selection, which may be performed prior to positive cell selection, antibodies bind to antigens that are expressed on contaminating cells, and used to deplete the biological fluids of non-tumor cells. In positive cell selection, antibodies bind to

10   tumor-associated antigens, and this binding is used to separate tumor cells from the biological fluids. When tumor cells are separated by means of antibody-coupled magnetic beads, cells can be released from the beads by digestion of the antigen/antibody binding sites with chymopapain or by other means. The resulting separated tumor cells can re-express the tumor-associated antigen after a short time in

15   culture. The tumor cells are expected to contribute genes encoding known and unknown tumor-associated antigens to the hybridoma of the invention.

Tumor cells can also be separated from solid tissue samples, using a combination of physical, enzymatic and immunological methods. Macroscopic peri-

20   tumoral stromal tissue can be removed by dissection prior to reduction of the tumor to a cell suspension. Density centrifugations and antibody-mediated separations can then be performed on the cell suspension as described above.

(2) The purified tumor cells are then prepared for cell fusion. Three types of

25   tumor partners can be prepared: (i) primary cultured tumor cells, (ii) immortal tumor cells, and (iii) drug-sensitive immortal tumor cells. Primary cultured tumor cells are purified tumor cells which have been cultured for a limited period of time in the presence of appropriate growth factors. Immortal tumor cells are permanent cell lines derived from these primary cultured tumor cells; such permanent cell lines can be

30   obtained, for instance, after culturing the primary tumor cells for longer periods of time

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in the presence of appropriate growth factors, or by transducing the primary tumor cells with immortalizing genes. Finally, drug-sensitive immortal tumor cells are permanent cell lines derived from spontaneous mutants of immortal tumor cells; these mutants are selected by culturing the immortal tumor cells in the presence of an appropriate drug. These drug-sensitive immortal tumor cells die when they are exposed to the drug to which they are sensitive. For example, 6-thioguanine was used to select the murine P815* mastocytoma cell line described in Example 1, and 5-bromo-2'-deoxyuridine was used to select the human 143B osteoasarcoma cell line described in Example 7. Both cell lines die when cultured in HAT-containing medium, as described in Examples 3 and 9.

(2a) As an alternative to step (2) a pre-established immortal human tumor cell line can be used, provided that at least one of the tumor-associated antigens from the patient's tumor cells are matched to these pre-established immortal tumor cells.

(3) A sample is provided with a source of DLCs. Such samples include for example peripheral blood, cord blood, bone marrow, lymph or accessible lymph nodes; they may be taken from the patient or from a healthy, HLA-compatible donor. From there, two alternatives are available. Functionally-competent DLCs can be purified directly from these samples, using various methods described in the literature. Alternatively, functionally-competent DLCs can be purified after in vitro differentiation of the precursors contained in these samples, which can be done by culturing the latter in the presence of cytokines, as described hereunder.

(4) The DLCs are prepared for cell fusion, in one of the 4 following ways. (1°) Primary DLCs purified directly from blood, lymph or other tissues are maintained in culture for no longer than 24 hours, as described for mouse spleen DLCs in Example 2. (2°) Primary cultured DLCs differentiated from blood, bone marrow or other tissues are cultured for at least 7 days in the presence of cytokines, as described for human blood DLCs in Example 8 or as published by Sallusto and Lanzavecchia, 1994, J. Exp.

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Med. 179: 1109-111; Romani et al, 1994, J. Exp. Med. 180: 83-93; Mackensen et al,

1995, Blood, 86: 2699-2707. (3°) Immortal DLCs can be derived from primary-

cultured DLCs, for example by adapting the method described by Paglia et al, 1993, J.

Exp. Med. 178: 1893-1901. These authors immortalized neonatal mouse spleen DLCs

5   by using a recombinant retrovirus. (4°) HAT-sensitive variants of these DLC lines can

thereafter be derived by standard culture techniques, to yield drug-sensitive immortal

DLCs.

(5) A tumor cell partner is then fused with a DLC partner. From there, two

10   alternatives are available, namely to separate or not to separate the fused cells by

metabolic selection. After fusion, the treated cells include a plurality of DLC/tumor

cell hybrids, as well as unfused tumor cells and unfused DLCs. If no selection is

applied, fused cells as well as unfused cells are used for inducing an anti-tumor

immunity in vivo and/or in vitro (see § 6). If a metabolic selection is applied, for

15   example by plating the treated cells in HAT-medium, only the immortal, HAT-resistant

hybrid cells survive (Examples 3 and 9) and permanent cell lines hereafter termed

DLC/tumor cell hybridomas are developed from them.

(6)   The DLC/tumor cell hybridomas with therapeutic potential are then

20   selected from all growing hybridomas. Their therapeutic potential is linked to the

retention of pertinent DLC characteristics and of pertinent tumor cell characteristics.

Pertinent DLC characteristics include DLC morphology, DLC surface markers, DLC

genetic markers and the capacity to activate immune cells in vitro. At least one of

these DLC characteristics may suffice to qualify hybridomas made of (drug-sensitive)

25   immortal tumor cells and primary cultured DLCs, since these hybridomas necessarily

inherited immortality from the tumor parent. (1°) The selection may be based on the

morphologic DLC appearance of the hybridoma by scanning electron microscopy

(SEM), as shown in Example 4A and Figure 1. Such an analysis can be performed on

a minute sample of cells at a very early stage of hybridoma development, allowing the

30   culture efforts to be focused on the dendritic-like hybridomas. (2°) In the absence of

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morphological DLC characteristics, as in Example 10A, the expression of DLC surface markers may be used to select hybridomas with therapeutic potential. If such DLC surface markers, including namely T-cell activating molecules, are not expressed on resting hybridomas, they may nevertheless be induced by treatment with cytokines or

5   other activating agents, as described in Example 10B. (3°) Genetic DLC markers are further used to confirm or to exclude the contribution of a T-cell, B-cell or other cell type to the hybridoma, as in Examples 4C and 10C. HLA-DR gene typing can also be used to identify blood donor genes when the tumor cell and the DLC are from distinct individuals, as in Example 10C.

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In DLC/tumor cell hybridomas involving patient's related pre-established immortal tumor cells, it is necessary to select dendritic-like hybridomas that express in addition at least one of the patient's matched tumor-associated antigens. Standard immunocytochemistry can be performed on small samples of the hybridomas to identify

15   such tumor-associated antigens as Her2/neu for breast cancer and carcinoembryonic antigen (CEA) for colon cancer. The hybridomas identified as potentially useful are amplified in culture for complete phenotypic characterization (chromosomes, genetic markers, cell surface markers and sub-cellular morphology) and for clinical use.

20   The thirteen embodiments of our invention are briefly described as follows:

Embodiments A, B, C: primary cultured patient's tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (A), or with primary cultured DLCs differentiated from precursors derived from blood, bone marrow or other tissue (B), or with immortal DLCs (C), to yield a plurality of DLC/tumor cell

25   hybrids that are are used without selection.

Embodiments D, E: primary cultured patient's tumor cells are fused with immortal DLCs (embodiment D) or with drug-sensitive immortal DLCs (embodiment E) to yield a plurality of DLC/tumor cell hybridomas; the latter are mixed in embodiment D with unfused immortal DLC. In these embodiments, hybridomas with

30   both DLC characteristics and tumor cell characteristics may be selected for further use.

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Embodiments F, G: patient's immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (F), or with primary cultured DLCs differentiated from precursors (G), to yield a plurality of DLC/tumor cell hybridomas, mixed with unfused immortal tumor cells. In these embodiments,

5   hybridomas with DLC characteristics are selected for further use.

Embodiments H, I: patient's drug-sensitive immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (H), or with primary cultured DLCs differentiated from precursors (I), to yield a plurality of DLC/tumor cell hybridomas. In these embodiments, hybridomas with DLC

10   characteristics are selected for further use.

Embodiments J, K: Patient's related, pre-established immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (J), or with primary cultured DLCs differentiated from precursors (K), to yield a plurality of DLC/tumor cell hybridomas, mixed with unfused immortal tumor cells. In these

15   embodiments, hybridomas with DLC characteristics and expressing in addition the patient's matched tumor-associated antigen(s) may be selected for further use.

Embodiments L, M: Patient's related, pre-established, drug-sensitive immortal tumor cells are fused with primary cultured DLCs purified from blood, lymph or other tissue (L), or with primary cultured DLCs differentiated from precursors (M), to yield

20   a plurality of DLC/tumor cell hybridomas. In these embodiments, hybridomas with DLC characteristics and expressing in addition the patient's matched tumor-associated antigen(s) may be selected for further use.

(7) The selected hybridomas are then used for inducing an anti-tumor immunity, either in vivo or in vitro, thereby contributing to the rejection of the residual

25   tumor in the patient. For the induction of an anti-tumor immune response in vivo, the DLC/tumor cell hybridomas are irradiated or otherwise inactivated, and injected, for example sub-cutaneously, into the patient. The patient is monitored for signs of an anti-tumor immune response and for the clinical evolution of his/her cancer. In a murine model, a single injection of a living DLC/tumor cell hybridoma into syngeneic

30   mice elicited an anti-tumor immune response as shown in Examples 5A and 5B. In

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addition, multiple injections of an irradiated DLC/tumor cell hybridoma had a therapeutic effect on mice preinoculated with a lethal dose of tumor cells, as shown in Example 5C. For the induction of an anti-tumor immune response in vitro, the DLC/tumor cell hybridomas are irradiated or otherwise inactivated, and cultured with
5   the immune cells of the patient. The activated immune cells are then re-injected into the patient. The patient is monitored for the presence of an anti-tumor immune response and for the clinical evolution of his/her cancer.

Herein, the term "dendritic-like cell (DLC)" is an operational term refering to a
10   non-B cell present in preparations of purified or enriched dendritic cells. DLCs can be dendritic cells of myeloid origin, monocytes, cells intermediate between dendritic cells and monocytes, T-cells or other non-B cells present in the preparation.

Herein, the term "DLC/tumor cell hybrid" is defined as a fused cell which
15   exhibits characteristics of both a DLC and the specific tumor cell of interest. Since a DLC may be a dendritic cell, a monocyte, a T-lymphocyte or another non-B cell co-purifying with dendritic cells, DLC/tumor cell hybrids may include hybrids with different phenotypic characteristics reflecting these different cell fusion partners. A plurality of DLC/tumor cell hybrids is capable of eliciting an immune response, either
20   in vivo or in vitro, against the tumor fusion partner which makes up part of the genome of the hybrids. This capacity is not inhibited by the presence of unfused DLCs, DLC lines or unfused tumor cells or tumor cell lines.

Herein, the term "DLC/tumor cell hybridoma" is defined as an immortal hybrid
25   cell line, which exhibits characteristics of both a DLC and the specific tumor cell of interest. Since a DLC may be a dendritic cell, a monocyte, a T-lymphocyte, and other non-B cells co-purifying with dendritic cells, DLC/tumor cell hybridomas may exhibit phenotypic characteristics of any of these cell lines. For instance, in examples below, 2 murine DLC/tumor cell hybridomas exhibited T-cell lineage characteristics, whereas 1
30   human DLC/tumor cell hybridoma was likely from monocytic origin. More

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importantly, a DLC/tumor cell hybridoma is capable of eliciting an immune response, either _in vivo_ or _in vitro,_ against the tumor fusion partner which makes up part of the genome of the hybridoma.

5   Herein, the term "anti-tumor response _in vivo_" refers to the _in vivo_ induction of immune effectors that confer resistance to a subsequent challenge with tumor cells, and contribute to the rejection of pre-existing tumor cells. In Example 5B, these immune effectors include cytotoxic T-lymphocytes that were detected by submitting the spleen cells of the immunized animals to an _in vitro_ assay. In human subjects, appropriate

10   non-invasive measures can be used for demonstrating the presence of anti-tumor immune effectors. However, the clinical course of the tumor, monitored by imaging techniques and the survival of the patient, will be the prime criterion for the evaluation of the immunotherapy.

15   Herein, the term "anti-tumor response _in vitro_" refers to the _in vitro_ activation of autologous immune cells into anti-tumor immune effectors. The latter will contribute to the rejection of the pre-existing tumor cells when infused into the patient. The secretion of IL-2 by the murine T-DLC/tumor cell hybridomas (Example 6) and the secretion of GM-CSF by the human (presumed monocytic) DLC/tumor cell

20   hybridoma may contribute to such _in vitro_ and _in vivo_ activation of anti-tumor immune cells.

          Herein, the term "DLC characteristics" shared by the hybridoma of the invention refers to DLC morphology, the expression of DLC surface markers, the

25   expression of DLC genetic markers and/or the activation of immune cells.

          Herein, the term "DLC morphology" refers to a typical image observed by scanning electron microscopy. The images of the DLC/tumor cell hybridoma are compared to those of the parent tumor cell and DLC. At first glance, to one skilled in

30   the art, it is clear that the hybridoma resembles the DLC more than the tumor cell.

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Upon analysis, DLCs have irregular shapes, due to the presence of clearly-visible, flat cytoplasmic extensions like pseudopodia and veils. Hybridomas with such similar cytoplasmic extensions can be recognized as having a dendritic-like cell morphology, as illustrated in Fig. 1 (see Example 4). These data are also consistent with the

5 possibility that other embodiments of the present invention may express these or other DLC morphological traits, since the DLC morphology of a DLC/tumor cell hybridoma is expected to mirror the particular morphology of the DLC used as a fusion partner.

Herein, the term "expression of DLC surface markers" refers to the expression

10 of markers restricted to the DLCs used for fusion. These markers include T-cell activating molecules and other molecules. T-cell activating molecules are expressed on activated APCs; they include mainly MHC class I and class II restricting elements, as well as the family of B7 costimulatory molecules; the latter bind to the CD28/CTLA4 counter-receptor on T-cells. Other DLC surface markers include, for example, CD1a

15 for human myeloid dendritic cells, CD14 for monocytes, and the TCR/CD3 complex for T-cells. It is shown in Example 4B (Table 1 and Figure 2) that the HY41 and HY62 hybridomas express MHC class I molecules and the TCR/CD3 complex, but neither MHC class II molecules, nor B7 costimulators. When such T-cell activating molecules are not expressed on resting hybridomas, they can sometimes be induced by

20 exposure to cytokines or other activating agents; Example 10B illustrates such an induced expression of HLA-DR on a human DLC/tumor cell hybridoma.

Herein, the term "tumor-associated antigen" refers to a peptide derived from a protein expressed by a tumor cell which, when expressed by the hybridoma of the

25 invention, will enable the hybridoma to elicit a tumor-specific response in vivo and/or in vitro . It also refers, by extension, to the proteins from which the antigenic peptides are derived, and to the genes encoding the antigenic proteins.

Herein, the term "activation of immune cells in vivo" refers to the immune

30 rejection of a residual tumor, as measured by its reduction in size and by the survival of

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the patient, as shown for mice in Example 5C. In vitro correlates of this in vivo state of immunity include for example the detection of blood or tissue immune cells able to kill the patient's own tumor cells in vitro. In experimental animals, the quoted expression also refers to the immune rejection of the living hybridoma, to the immune

5   resistance to a subsequent inoculation of tumor cells, and to the presence of tumor-specific cytolytic effector cells in the lymphoid organs of the tumor-resistant animals, as shown in Example 5.

Herein, the term "activation of immune cells in vitro" refers for example to a

10   mixed lymphocyte-tumor cell reaction, wherein the dendritic cell/tumor cell hybridoma ("the tumor cell") stimulates one of the following reactions by allogeneic T-cells ("the lymphocyte"): (1) T-cell proliferation, as measured by tritiated thymidine incorporation; (2) T-cell secretion of cytokines including for example IL-2, interferon-gamma and others, as measured by ELISA, bioassay, or reverse transcription

15   polymerase chain reaction; (3) T-cell-mediated tumor cell lysis, as measured by chromium release assay. This term may also refer to the activation of other immune cells, like monocytes and natural killer cells, and can be measured, for example, by cytokine release or cytotoxic cell assays.

20   The following experimental examples are provided to illustrate the invention.

<u>Example 1</u>

Preparation of Murine Tumor-Derived Cells.

25

    The P815-X2 cell line was derived from the methylcholanthrene-induced mastocytoma P815 of mouse DBA/2 origin (Dunn and Potter, 1957, J. Natl. Cancer Inst. 18: 587-601. This cell line was obtained by Thierry Boon, director of the Ludwig Institute for Cancer Research, Brussels Branch, Belgium, and recloned by his group

30   (Uyttenhove et al, 1980, J. Exp. Med 1562: 1175-1183). The subclone P1 was extensively used by T. Boon's group and given to the present inventors in 1980. A 6-thioguanine-resistant mutant was derived from P1, as described by Le et al, 1982, Proc. Natl. Acad. Sci. USA 79:7857-7861. Briefly, P1 cells were cultured in

Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Merelbeke, Belgium), in a 7% CO2 atmosphere. Increasing concentrations of 6-thioguanine (Sigma, Bornem, Belgium), ranging from 1 µg/ml to 30 µg/ml were added to the
5   culture. The final 6-thioguanine-resistant cells died in HAT-medium, i.e. in medium supplemented with 10^{-4} M hypoxanthine, 3.8×10^{-7} M aminopterin, and 1.6×10^{-5} M 2-deoxythymidine (HAT supplement, Gibco BRL). Several HAT-sensitive clones were isolated by limiting dilution from these 6-thioguanine-resistant cells. A HAT-sensitive clone expressing MHC class I antigens was used in the present invention and will
10 hereafter be called P815*.

P815* cells were cultured at 37°C in a 7% CO2 atmosphere in tissue culture flasks (Becton Dickinson, CA) containing RPMI 1640 medium (Seromed Biochem KG, Berlin, Germany) with 10% FCS (Gibco BRL). One day before use, P815* cells
15 were diluted with fresh medium in order to be in exponential growth phase at the time of cell fusion.

Example 2

20 Preparation of Murine Dendritic-Like Cells from the Spleen

The preparation of splenic DLCs was done according to a multi-step procedure initially described by Crowley et al, 1989, Cell. Immunol. 118: 108-125. This procedure was adapted as described by Sornasse et al, 1992, J. Exp. Med 175:15-21.
25 The procedure was started one day before the fusion experiment and yielded 200,000 to 500,000 DLCs per spleen.

Briefly, DBA/2 mice were obtained from Charles River, Sulzfeld, Germany, and maintained in specific pathogen-free conditions. Animals 8 to 10 weeks old were
30 killed by cervical dislocation; their spleens were quickly removed and kept in cold RPMI 1640 medium. The spleens were digested with collagenase (CLSIII; Worthington Biochemical Corp., Freehold, NJ) and separated into low and high density fractions on a bovine serum albumin gradient (Bovuminar Cohn fraction V

powder; Armour Pharmaceutical Co., Tarrytown, NJ). Low-density cells were cultured during 2 hours in RPMI 1640 medium with 10% FCS, and the non-adherent cells were removed by vigorous pipetting. The latter were further cultured for 1 hour in serum-free RPMI 1640 medium. The non-adherent cells were removed by gentle pipetting and cultured overnight in RPMI 1640 medium with 10% FCS. The final non-adherent fraction contained at least 95% dendritic cells, as assessed by morphology and specific staining.

<u>Example 3</u>

Fusion of Murine Tumor Cells and Dendritic-Like Cells

The procedure used to fuse HAT-sensitive tumor cells with mortal splenic DLCs was adapted from procedures used in our laboratory to generate monoclonal antibodies, as described by Franssen et al, Protides of the Biological Fluids, editor H. Peeters, Pergamon Press, Oxford, 1982, pp 645-648.

Briefly, splenic DLCs and P815* cells were extensively washed in serum-free RPMI 1640 medium. Five million DLCs were mixed with the same number of HAT-sensitive P815* cells in a 15 ml conical tube and centrifuged. Two hundred μl of a 50% solution of polyethylene glycol (PEG 4000, Merck AG, Darmstadt, Germany) in RPMI 1640 medium were added dropwise to the cell pellet. The fusion was then stopped by the stepwise addition of RPMI 1640 medium.

The cells were washed to remove the PEG and resuspended in RPMI 1640 medium with 10% FCS. After 2 hours incubation at 37°C, the cells were centrifuged, resuspended in RPMI 1640 medium containing HAT and 10% FCS, and plated at 10^4 cells/well in flat-bottomed 96-well plates (Becton Dickinson, CA). The plates were seeded one day before use with a feeder layer consisting of 5,000 irradiated peritoneal cells/well. Peritoneal cells were taken from Balb/c mice and irradiated at 2,000 rads from a Cobalt 60 source before plating. The plated fusion was cultured at 37°C in a 7% CO_2 atmosphere. The medium (RPMI 1640 with 10% FCS and HAT) was renewed as required by cell growth. In these conditions, unfused DLCs, that are not

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immortal, died within a few days of culture; unfused P815* cells, that are immortal but HAT-sensitive, died in the HAT-containing-medium, and only hybrid cells, combining the immortality of P815* cells with the HAT-resistance of DLCs survived and developed into growing DLC hybridomas.

5   After 3-4 weeks of culture, wells that contained a growing DLC hybridoma could be clearly identified by phase contrast microscopy. The content of a positive well was transferred into a larger well (24-well plates, Becton Dickinson, CA) previously seeded with irradiated peritoneal cells. Eventually, DLC hybridomas were

10   transferred to small tissue culture flasks (Becton Dickinson, CA) and amplified for characterization and storage in liquid nitrogen.

Example 4:

15   Selection of Murine Dendritic-Like Cell/Tumor Cell Hybridomas with Therapeutic Potential

The goal of these experiments was to select DLC/tumor cell hybridomas exhibiting at least one of the three following characteristics: (1°) a DLC morphology; (2°) DLC surface markers; (3°) DLC genetic markers.

20

A. Dendritic-Like-Cell Morphology

          P815* tumor cells, fresh splenic DLCs, and DLC/tumor cell hybridomas were analyzed by scanning electron microscopy (SEM). About one million cells were fixed

25   in 2-4% glutaraldehyde for 24 hours at room temperature and washed in phosphate buffer saline. Cell suspensions were then collected on 0.2μM nylon filters, postfixed in 1% osmium tetroxide followed by 1% tannic acid mordant and uranyl acetate, with a series of saline washes in between each step. The samples were dehydrated through graded alcohols, then critical point dried from CO_2. After critical point drying, the

30   samples were mounted on aluminium stubs and sputter coated with gold using a Bio-Rad PS3 coating unit. The cells were examined at 20 kV in a Hitachi S520 scanning electron microscope.

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Photographs of the cells are shown in Fig. 1. In these conditions, P815* tumor cells appeared as uniform rounded cells, whose surface was spiked with numerous short microvilli (Fig. 1a). In contrast, splenic DLCs appeared as irregular cells, due to

5   the presence of clearly-visible cytoplasmic extensions, resembling pseudopodia and veils. Furthermore, the DLC surface was not spiked with numerous microvilli, but displayed instead fewer, larger protrusions. The hybridoma cells were in general much larger than the parent P815* cells. Many of them (like the one named HY1) looked very much like the P815* parent, which was linked to their round regular shape and

10   microvilli-like protrusions (Fig. 1c). In contrast, hybridomas HY41 and HY62 looked much more like the DLC parent, when considering their irregular shape and relatively bare cell surface with some large protrusions, as shown for HY41 in Fig. 1d. However, a DLC morphology may be assumed not only by dendritic cells of myeloid origin, but also by cells derived from other lineages, including cells of the B- and T- lymphocyte

15   lineages, like follicular dendritic cells and dendritic epidermal T-cells, respectively. In order to determine the cell lineage of the DLC that fused with the P815* tumor cell, other DLC characteristics were investigated for hybridomas HY41 and HY62.

B. Dendritic-Like-Cell Surface Markers

20   Cell surface molecules were characterized by FACS analysis, as described by Flamand et al, 1990, J. Immunol 144:2875-2882. Briefly, the cells were preincubated with 2.4G2, a rat anti-mouse Fc-receptor (Fc-R) monoclonal antibody (mAb) for 10 min prior to staining with fluorescein-coupled monoclonal antibody (fl. mAb). This preincubation was done to prevent the non-specific binding of mAb to cellular Fc-R.

25   When unlabelled mAb were used, they were revealed by incubation with fluoresceinated anti-IgG antibodies. The labelled cells were gated for size and side scatter to eliminate dead cells and debris, and analyzed on a Facscan (Becton Dickinson, CA).

23

The results are summarized in Table 1. No T-cell activating molecules or other dendritic-cell-associated molecules were expressed by the HY41 and HY62 hybridomas. However, a fraction of the cells of both hybridomas expressed surface CD3ε chains of the T-cell receptor (TCR), suggesting that they were T-lymphocyte/tumor cell hybridomas. After cloning by limiting dilution, CD3+ and CD3- subclones were isolated from both hybridomas. Figure 2 shows that the HY41 and HY62 CD3ε+ subclones were also labeled by a fl mAb specific for the V β8 domain of the TCR, whereas P815* tumor cells and the CD3ε-subclones remained unstained. These results showed that the HY41 and HY62 hybridomas expressed an α/b TCR, and hence had incorporated a dendritic-like T-lymphocyte. However, neither CD4 or CD8 were expressed by the hybridomas. In order to confirm these cell surface marker studies, genetic marker studies were undertaken.

24

Table 1. Cell Surface Markers of Murine Dendritic-Like-Cell/Tumor Cell Hybridomas
HY41, HY62 and Parent Cells

Reagents	Surface markers	DLCs[1]	P815*	HY41	HY62
	Present on DLCs and P815*				
31.3.4 mAb	MHC class I Kd	+	+	+	+
34.4.20 mAb	MHC class I Dd	+	+	+	+
30.5.7 mAb	MHC class I Ld	+	+	+	+
3E2 fl mAb	ICAM-1 (CD54)	+	+	-	-
	Present on P815* only				
2.4G2 mAb	Fc-R	-	+	-	-
	Present on DLCs only				
	T-cell activating molecules:				
14.4.4 fl.mAb	MHC class II	+	-	-	-
16-10A1 fl mAb	B7-1 (CD80)	+	-	-	-
GL1 fl mAb	B7-2 (CD86)	+	-	-	-
CTLA4- human Ig	CTLA4-ligand	+	-	-	-
M1/69 fl mAb	HSA (CD24)	+	-	-	-
	Other molecules:				
N418 fl mAb	N418 (CD11c)	+	-	-	-
145-2 C11	CD3ε	nd[2]	-	+	+
F23-1	TCR V β8 chain	nd	-	+	+
H129.19	CD4	nd	-	-	-
53-6.7	CD8a	nd	-	-	-

[1]: By cell scatter and cell surface marker analyses, DLCs contained more than 95% dendritic cells;
[2]: nd: not detectable
31.3.4, 34.4.20, 30.5.7: mouse anti-mouse H2-K^d, D^d and L^d mAb, respectively; Ozato et al, 1980, J.
    Immunol. 124:533-;
3E2: hamster anti-ICAM-1, from Pharmingen, San Diego, CA
2.4G2 : rat anti-mouse Fc-gamma-RII/III mAb (Unkeless, 1979, J. Exp Med   150:580-586;
14.4.44: mouse anti-I-E^d fluorescein-coupled mAb (fl mAb);Ozato et al, 1980, J. Immunol. 124:533-
16-10A1: rat anti-B7-1 fl mAb; Razi-Wolf et al, 1993, Proc. Natl. Acad. Sci. USA 90:11182-11186;
GL1: hamster antiB7-2 fl mAb;Hathcock et al, 1993, Science 262:905-907;
CTLA4-human IgG fusion protein: Linsey et al, 1991, J. Exp. Med 174:561-569;
M1/69: Rat anti-HSA, from Pharmingen, San Diego, CA.
N418: hamster anti-mouse CD11c; Metlay et al, 1990, J. Exp. Med 171:1753-1771;
145-2C11: hamster anti-mouse DC3ε fl mAb; Leo et al, 1987, Proc.Natl.Acad.Sci.USA 84:1374
F23.1: mouse anti-mouse TCR V β8 fl mAb from ATCC, Bethesda MD.
H129.19: rat anti-mouse CD4 fl mAb, from Gibco BRL, Gaithersburg, MD.
53-6.7: rat anti-mouse CD8a fl mAb, from Gibco BRL, Gaithersburg, MD.
ND: not detectable

25

C. DLC Genetic Markers

First, Southern blot analysis was used to analyse the rearrangement status of the TCR genes in genomic DNA from the HY41 hybridoma. The mouse T-cell hybridoma 13.26.8-H6 was used as a reference for rearranged TCR genes (Ruberti et al, 1992, J. Exp. Med. 175: 157-162), and P815* mastocytoma cells as well as DBA/2 spleen cells were taken as controls for germ line TCR genes. Genomic DNA was extracted from 2 x 10^7 cultured cells and from spleens, using the Genome DNA Kit (Bio 101, CA, USA) according to the manufacturer's instructions. Ten μg of DNA were digested for ± 4 hours with various restriction enzymes, separated on a 1% agarose gel and transferred to a nylon membrane (Qiabrane Nylon plus, Qiagen, Hilden, Germany) according to standard procedures. The blot was hybridized to a DIG-labeled synthetic oligonucleotide of 50 bases targeted to the first exon of the constant region of the mouse TCR β chain and processed for chemiluminescent detection using Boehringer Mannheim's DIG detection kit. The results showed that the HY41 genome contained a rearranged TCR β chain gene, which is a hallmark of T-cell lineage commitment (not shown).

Next, the Polymerase Chain Reaction (PCR) was used to detect rearranged V β8-Cβ sequences of the TCR in genomic DNA. The upstream primer was targeted to bases 47-66 with respect to the ATG initiation codon of the mouse V β8 region (5'-AACACATGGAGGCTGCAGTC-3') and the downstream primer was targeted to bases 141-160 of the fisrt exon of the Cβ region (5'-GTGGACCT CCTTGCCATTCA-3'). The PCR was carried out essentially according to the instructions of Boehringer Mannheim's Long Range Expand PCR System. Analysis of the PCR products on a 1% agarose gel stained with ethidium bromide is shown in Figure 3. A fragment with the expected length (4.5 to 5 kb)of the rearranged Vβ8-Cβ fragment is clearly seen in DNA from the T-cell hybridoma 13-26-8-H6 (lane T), used as a positive control, as well as in DNA from the HY41 and HY62 hybridomas (lanes 41 and 62); this fragment is not amplified in DNA from P815* tumor cells and from spleen cells (lanes P and S), used as negative controls. These results confirm that the

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DLC that fused with a P815* tumor cell to yield the HY41 and HY62 hybridomas was a T-lymphocyte expressing an α/β TCR receptor, including the Vβ8 domain. These hybridomas will hereafter be termed T-DLC/tumor cell hybridomas.

5   In conclusion, the HY41 and HY62 T-DLC/tumor cell hybridomas were selected for further studies because of their DLC morphology and T-lymphocyte lineage. In both hybridomas, the T-lymphocyte fusion partner was a rare and undetectable contaminant of the splenic DLC preparation. In view of the complex genetic regulations controling CD4 and CD8 expression in somatic cell hybrids 10   (Wilkinson et al, 1991, J. Exp. Med. 174: 269-280), it is impossible to determine a posteriori if the fusing T-cell was a CD4+, CD8+, or CD4-CD8- "double negative" T-cell. However, whatever the sublineage of T-lymphocyte involved, the next step was to determine the in vivo immunogenicity of these T-DLC/tumor cell hybridomas.

15   Example 5

In vivo Immunogenicity of Murine T-Dendritic-Like-Cell/Tumor Cell Hybridomas

The goal of these experiments was to determine if the hybridomas induced an efficient 20   immune rejection in vivo, as measured by the following criteria: (1°) rejection of the hybridomas by immunocompetent mice; (2°) vaccination with the hybridomas against a subsequent inoculation of tumor cells; (3°) treatment with the hybridomas after prior inoculation of tumor cells.

25   A. Immune Rejection of T-DLC/Tumor Cell Hybridomas
        Groups of 10 to 12 DBA/2 mice were injected intra-peritoneally with 500,000 living cells of the P815* tumor or of the HY41 hybridoma. Injected animals included mice immunosuppressed by sub-lethal irradiation as well as immunocompetent mice. All irradiated animals died from their tumor within four weeks of inoculation, showing 30   that the HY41 and P815* cell lines were very similar in their tumorigenicity (Fig. 4). In contrast, 9/12 (75%) immunocompetent animals injected with the HY41 hybridoma survived two months after inoculation, when only 2/10 (20%) mice had survived the

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parental tumor injection. This experiment showed that the HY41 hybridoma was as tumorigenic as the parent tumor in irradiated mice, but more immunogenic than P815* in immunocompetent mice. Similar results were obtained with hybridoma HY62 (not shown).

5

B. Induction of Tumor Resistance by Murine T-DLC /Tumor Cell Hybridomas

The 9 surviving HY41-treated mice, as well as 9 untreated animals, were challenged intra-peritoneally with 500,000 P815* cells. All (9/9) untreated mice died from their tumor within six weeks of inoculation, showing that the tumor cell injection

10   was lethal for unimmunized animals. By contrast, 7/9 HY41-treated animals were still alive 6 weeks after tumor challenge, and 4/9 of them survived for at least three months (Fig. 5). These results strongly suggested that prior treatment of syngeneic mice with living HY41 DC hybridoma cells induced a memory immune response against the parent P815* cell line, conferring tumor resistance to 44% of the treated animals. A

15   similar tumor-resistance could be induced by the injection of living HY62 hybridoma cells (not shown).

The spleens of the 4 P815*-resistant mice were tested in vitro for the presence of anti-P815* cytotoxic T-cells, as described by Moser et al, 1987, J. Immunol 138:

20   1355-1362. Briefly, spleen cell suspensions were stimulated in vitro during 5 days with the irradiated P815* cells, in order to induce a measurable memory response. They were then used as effector cells on chromium-loaded P815* and L1210 target cells. The latter have the same MHC class I haplotype (H-2d) as P815 cells. At several effector/target ratios, the spleen cells of the untreated animals completely failed

25   to lyse the P815* and the L1210 target cells (Figs 6A and 6B). In contrast, the spleen cells from the 4 P815*-resistant mice lysed efficiently and specifically the P815* targets, without showing any significant activity on the L1210 targets. These results showed that the HY41-treated, P815*-resistant animals were able to mount a strong and tumor-specific cytolytic response upon in vitro restimulation.

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C. Induction of Tumor Treatment by Murine T-DLC/Tumor Cell Hybridomas.

In this experiment, 40 DBA/2 mice received an ip injection of 2×10^5 P815* tumor cells. Seven days later, the mice were divided into 4 groups of 10 animals; the first

5   group was left untreated while the 3 other groups were treated by 4 weekly ip injections of 2×10^6 irradiated (15,000 F) P815*, HY41 or HY62 cells. The data are presented in figure 7. Untreated animals all died within 7 weeks of tumor inoculation, and 20% of the mice treated with irradiated P815* tumor cells survived, confirming the weak immunogenicity of the P815 tumor cell line. In contrast, 60% and 40% of

10   the mice treated with irradiated HY41 and HY62 hybridoma cells, respectively, survived the prior injection of a lethal dose of P815* cells. These data showed that hybridoma HY41, and to a lesser extent hybridoma HY62, could induce the immune rejection of an established tumor. However, the mechanism leading to such an efficient in vivo immune rejection remained unclear.   One possibility that was explored

15   concerned the secretion of immunomodulating cytokines.

Example 6:

In vitro analysis of cytokine expression by T-DLC/tumor cell hybridomas

20   The goal of these experiments was to determine whether the HY41 and HY62 hybridomas synthesized some cytokines that could account, at least in part, for their in vivo immunogenicity. Total RNA was prepared from activated spleen cells, from P815* tumor cells and from the HY41 and HY62 hybridomas according to standard procedures. The Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and

25   cytokine-specific primers were used to amplify IL-2, IL-4, IL-10 and interferon γ (IFNγ) mRNA sequences, as described by De Wit et al, J. Immunology, 1993, 150: 361-366.   The primers used to amplify IL-12 p40 sequences were 5'-TTCAACATCAAGAGCAG TAGC-3' and 5'-GGAGAAGTAGGAATGGGGAGT-3'. Analysis of the RT-PCR products on ethidium bromide-stained agarose gels

30   showed that P815* tumor cells constitutively expressed IL-4 mRNA and that the HY41 and HY62 hybridomas constitutively expressed IL-2 and IL-4 mRNAs, but not

IL-10, IL-12, and IFNγ mRNAs. These cytokine mRNAs were nevertheless detected in activated spleen cells, used as a positive control. In conclusion, these data showed that the HY41 and HY62 T-DLC/tumor cell hybridomas constitutively expressed IL-4 like the parent P815* tumor cell, and IL-2, like the parent T-lymphocyte. These

5   cytokines, if secreted in vivo, may at least partially contribute to the immunogenicity of the hybridomas.

Example 7

Preparation of Human Tumor-Derived Cells

10   The human 143B thymidine kinase negative osteosarcoma cell line (hereafter termed 143B) is a HAT-sensitive cell line that was purchased from the ATCC (CRL n° 8303). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2% penicillin/streptomycin, 1% sodium pyruvate (all from Gibco BRL, Merelbeke, Belgium) and 0.015 mg/ml of 5-bromo-2'-deoxyuridine (Sigma Chemical

15   Co, St Louis, MO). One day before fusion, the cells were diluted with fresh medium in order to be in exponential growth phase.

Example 8

Preparation of human Dendritic-Like Cells from Peripheral Blood.

20   Dendritic cells were differentiated in vitro from adherent blood precursors, using an adaptation of the technique described by Romani et al, 1994, J. Exp .Med. 180: 83-93. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the buffy coat of a healthy donor by density gradient centrifugation on lymphoprep (Gibco BRL). Adherent cells were prepared by plating 10^7 PBMC on 6-well tissue

25   culture plates in 3 ml RPMI supplemented with 200 mM L-Glutamine, 50 µM Mercaptoethanol and 10% FCS. After 2 hours incubation at 37°C, the non-adherent cells were discarded by a very gentle rinse, and the adherent cells were further cultured in the above-described medium supplemented with GM-CSF (Leucomax, 800 U/ml) and IL-4 (Genzyme, 500 U/ml), at 37°C in a humidified atmosphere with 5% CO_2.

30   After 7 days of culture, DLCs were recovered and characterized by cell scatter and cell

surface marker analysis. The DLCs used for fusion contained 50% of monocytic-like cells, expressing CD14 but not CD1a or CD1c, as well as 38% of T lymphocytes, 4% of NK cells and 8% of B lymphocytes.

Example 9

Fusion of Human Tumor Cells and Dendritic-Like Cells

The procedure used to fuse HAT-sensitive tumor cells with DLCs was adapted from procedures used to generate monoclonal antibodies (Current Protocols in Immunology, chapter 2.5.4). The 143B tumor cells and the DLCs were extensively washed in serum-free medium (RPMI 1640); 2×10^6 DLCs were mixed with 1×10^6 143B osteosarcoma cells and centrifuged. The pellet was resuspended in 500 µl of a 50% solution of polyethylene glycol (PEG 4000, Gibco) in Dulbecco's phosphate buffered saline without Ca^{++}, Mg^{++} (ref. 14030035). After 1 minute, the PEG was progressively diluted by the slow and progressive addition of serum-free medium. The cells were washed free of PEG and resuspended in RPMI 1640 with 10% FCS. They were eventually plated at 2×10^4 cells/well in flat-bottomed 96-well plates (Falcon, Becton Dickinson) and cultured in a 5% CO_2 atmosphere at 37°C. HAT medium was added to the wells 24 hours after fusion and renewed every two days. In these conditions, unfused DLCs died within 2-3 weeks of culture, unfused 143B osteosarcoma cells died in HAT-medium and only hybrid cells combining the immortality of the tumor cell with the HAT-resistance of a DLC survived and developed into growing cell lines. After 3-4 weeks of culture, wells containing growing cell lines were clearly identified by phase contrast microscopy. Their contents were transferred into larger wells and eventually into culture flasks for amplification. Culture stocks were frozen in liquid nitrogen before analysis.

Example 10

Identification of Human Dendritic-Like Cells/Tumor Cell Hybridomas with Therapeutic Potential

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The goal of these experiments was to identify human DLC/tumor cell hybridomas presenting at least one of the three following characteristics: (A) DLC morphology; (B) DLC surface markers; (C) DLC genetic markers.

5   A. DLC morphology

The 143B osteosarcoma cells and a series of hybridoma cells were analyzed by SEM, as described in example 4. Comparison of the parent cells and hybridoma cells showed that none of the hybridomas analysed, including F3BG10 cells, displayed morphologic dendritic-like features. In the absence of such features, other dendritic-
10   like features were analyzed, namely the presence of DLC surface markers.

B. DLC surface markers

Cell surface markers were analysed as described in Example 4. Results are summarized in Table 2. None of the tested hybridomas, including F3BG10 cells,
15   expressed the T-cell activating molecules HLA-DR, B7.1, and B7.2. However, they expressed HLA class I, ICAM-1 (CD54) and LFA-3 (CD58), which were also present on the 143B tumor cells. They failed to express typical dendritic-cell markers like CD1a and CD1c, as well as markers specific for T-cells (CD3), B-cells (CD19), NK cells (CD56) and monocytes (CD14).

20

Since the hybridomas tested failed to express constitutively T-cell activating molecules, they were stimulated with a variety of cytokines in order to induce such expression. It was found that 40% of the F3BG10 hybridoma cells were induced to
· express varying amounts of surface HLA-DR after a 24 hour incubation with interferon
25   γ. After cloning by limiting dilution, subclones were tested for their capacity to express induced HLA-DR. Figure 8 shows that at least 90% of H12 cells clearly expressed induced HLA-DR, which greatly increases their immunogenic potential.

<center>32</center>

Table 2. Cell Surface Markers of Human Dendritic-Like-Cell/Tumor Cell Hybridoma

F3BG10 and Parent Cells

Reagents from	Surface markers	DLCs[1]	143B	F3BG10
	Present on DLCs and 143B			
Pharmingen	HLA class I	+	+	+
Immunotech	ICAM-1 (CD54)	+	+	+
Becton Dickinson	LFA-3 (CD58)	+	+	+
	Present on DLCs only			
	T-cell activating molecules:			
Becton Dickinson	HLA-DR	+	-	-[2]
Innogenetics	B7.1 (CD80)	+	-	-
Pharmingen	B7.2 (CD86)	+	-	-
	Other molecules:			
Immunotech	CD1a	+	-	-
Immunotech	CD1c	+	-	-
Becton Dickinson	CD14	+	-	-
Becton Dickinson	CD2	+	-	-
Becton Dickinson	CD3	+	-	-
Becton Dickinson	CD19	+	.-	-

[1]: By cell scatter and cell surface marker analyses, DLCs contained 50% monocytes, 38.% T-lymphocytes and 4% NK cells; the suspension also contained 8% of B-lymphocytes.

[2]: HLA-DR expression could be induced in 40% of F3BG10 cells and in 90% of its H12 subclone by incubation with interferon γ.

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C. DLC genetic markers

The goal of this first experiment was to determine whether the F3BG10 hybridoma had been generated by the fusion of a DLC with the 143B tumor cell, and to exclude that it was a revertant 143B tumor cell clone, that had become resistant to
5   HAT-medium by mutation. This was done by typing the HLA-DR genes of the blood donor, of the 143B tumor cell and of the F3BG10 hybridoma. Genomic DNA was prepared according to standard procedures from 143B tumor cells, from the PBMC of the blood donor and from F3BG10 hybridoma cells. These DNAs were submitted to a non-isotypic HLA-DR B oligotyping method, described for the typing of DR B 1, 3, 4,
10   5 alleles by Buyse et al. 1993, Tissue Antigens 41: 1-4. The polymorphic second exon of the corresponding genes was amplified by PCR, and biotinylated nucleotides were incorporated into the amplifying fragments during this procedure. The PCR products were hybridized with a combination of 31 sequence-specific oligonucleotide probes, immobilized in parallel lines on membrane strips. After a stringent wash, streptavidin-
15   labelled alkaline phosphatase was added to mark the biotinylated DNA fragments. The addition of the BCIP/NBT chromogen resulted in a colored precipitate. All reagents were part of the Innolipa DRB Key kit purchased from Innogenetics (Zwijndrecht, Belgium). The F3BG10 lane showed a mixture of bands corresponding to alleles present in the 143B osteosarcoma cells and in the PBMC of the blood donor,
20   confirming that F3BG10 hybridoma was a DLC/tumor cell hybridoma.

The goal of the second experiment was to investigate whether it was a T-lymphocyte or a B-lymphocyte that fused with a 143B tumor cell to yield the F3BG10 hybridoma. Genomic DNA was tested for the presence of rearranged T-Cell Receptor (TCR) genes or B-cell Receptor (BCR) genes by Southern blot analysis with TCR-
25   specific or BCR-specific probes. Standard procedures were used. Briefly, samples of 10 μg of DNA were submitted to overnight digestion at 37°C with different restricition enzymes. Hind3, Xba1 and Hind3 + Xba1 were used for the TCR rearrangements and EcoR1, Hind3 and Hind3 + BamH1 were used for BCR rearrangements. The
30   restriction fragments were separated by electrophoresis on a 1% agarose gel,

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transferred to nitrocellulose, baked and hybridized with probes specific for either the β chain gene of the TCR, or for a segment of the J gene of the Ig heavy chain of B lymphocytes. The results clearly showed that there were only germ line TCR genes and germ line BCR genes in the genomic DNA of the F3BG10 hybridoma. These data

5   excluded that the DLC/tumor cell hybridoma F3BG10 was produced by the fusion of the tumor cell with a T-lymphocyte or a B-lymphocyte. The DLC fusion partner could have been a monocyte, a dendritic cell, an intermediate cell between these two cells, a natural killer cell or another unidentified non-B cell. Because the pattern of cytokine secretion could provide indications on the cell lineage of the fusion partner, we

10 investigated cytokine secretion by F3BG10 cells.

Example 11

In vitro analysis of cytokine secretion by human DLC/tumor cell hybridoma

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The culture supernatants of the F3BG10 hybridoma cells and of the 143B tumor cells were assayed by ELISA for the presence of various cytokines, before and after 36 hours of culture in the presence of various stimuli including interferon γ, TNFα, GM-CSF and combinations of these. The results showed that the 143B

20 osteosarcoma cells and the F3BG10 hybridoma cells secreted similar levels of IL-6 and IL-8, that could be increased for both cytokines by stimulation with the above-mentioned cytokines. In addition, the F3BG10 cells but not the tumor cells secreted significant levels of GM-CSF, that could be increased by stimulation. Neither the tumor cells or the hybridoma cells secreted detectable levels of IL-1β, IL-10, IL-12

25 and TNFα. These results showed that the F3BG10 hybridoma secreted IL-6 and IL-8 like the parent tumor cell, and GM-CSF like the parent DLC. Since it was excluded that the latter was a T-lymphocyte, this result suggested that the fusion partner was a monocyte.

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What is claimed is:

1.   A plurality of dendritic-like cell/tumor cell hybrids which is capable of inducing an anti-tumor response.

2.   The plurality of hybrids of claim 1 which is capable of activating immune cells in vivo against a specific tumor.

3.   The plurality of hybrids of claim 1 which is capable of activating immune cells in vitro against a specific tumor.

4.   The plurality of hybrids of any one of claims 1-3 which is human in origin.

5.   A dendritic-like cell/tumor cell hybridoma which is capable of inducing an anti-tumor response.

6.   The hybridoma of claim 5 which is capable of activating immune cells in vivo against a specific tumor.

7.   The hybridoma of claim 5 which is capable of activating immune cells in vitro against a specific tumor.

8.   The hybridoma of any one of claims 5-7 which is human in origin.

9.   A method for producing a plurality of dendritic-like cell/tumor cell hybrids useful for the induction of an anti-tumor response, said method comprising;

        (a)   providing a sample of a tumor against which said response is needed,

        (b)   preparing a primary cell culture comprising tumor cells derived from said tumor sample,

        (c)   providing autologous or HLA-compatible allogeneic dendritic-like cells,

(d)   fusing said dendritic-like cells with said tumor cells to produce a plurality of hybrids.

10.   The method of claim 9 wherein the dendritic-like cells of step (c) are produced by culturing precursors in the presence of cytokines.

11.   The method of claim 9 wherein the dendritic-like cells of step (c) are members of an immortal cell line.

12.   A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising;
  (a)   providing a sample of a tumor against which said response is needed,
  (b)   preparing a primary culture of said tumor sample to provide tumor cells,
  (c)   deriving an immortal cell line from said tumor cells to produce immortal tumor cells,
  (d)   providing autologous or HLA-compatible allogeneic dendritic-like cells,

  (e)   fusing said dendritic-like cells with said immortal tumor cells to produce a plurality of hybridomas,
  (f)   selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

13.   The method of claim 12 wherein the dendritic-like cells of step (d) are produced by culturing precursors in the presence of cytokines.

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14. The method of claim 12 or claim 13 wherein the immortal tumor cells of step (c) are drug-sensitive, said method further comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

5 15. A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising:

    (a) providing a sample of a tumor against which said response is needed,

    (b) preparing a primary cell culture comprising tumor cells derived from said tumor sample,

10   (c) providing an immortal cell line comprising immortal autologous or HLA-compatible allogeneic dendritic-like cells,

    (d) fusing said immortal dendritic-like cells with said tumor cells to produce a plurality of hybridomas, and

    (e) selecting from said plurality of hybridomas a hybridoma which exhibits 15 at least one characteristic selected from the group consisting of tumor cell morphology, tumor cell surface markers, tumor cell chromosomal and genetic markers.

16. The method of claim 15 further comprising selecting from said plurality of hybridomas a hybridoma which exhibits at least one chracteristic selected from the 20 group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

17. The method of claim 15 or 16 wherein the dendritic-like cells of step (c) are drug sensitive, said method further comprising, after step (d), killing unfused drug-25 sensitive immortal dendritic-like cells by exposure to said drug.

18. A method for producing a dendritic-like cell/tumor cell hybridoma useful for the induction of an anti-tumor response, said method comprising:

    (a) providing a sample of a tumor against which said response is needed,

30   (b) analyzing tumor-associated antigens of said tumor sample,

(c)   providing an established cell line comprising immortal human tumor cells having at least one tumor-associated antigen in common with said tumor sample,

(d)   providing autologous or HLA-compatible allogeneic dendritic-like cells,

(e)   fusing said dendritic-like cells with said immortal tumor cells to produce

5   a plurality of hybridomas, and

(f)   selecting from said plurality of hybridomas a hybridoma which exhibits at least one characteristic selected from the group consisting of dendritic-like cell morphology, dendritic-like cell surface markers, dendritic-like cell genetic markers and immune cell activation in vitro.

10

19.   The method of claim 18 further comprising selecting from said plurality of hybridomas a hybridoma which expresses at least one tumor-associated antigen in common between the immortal tumor cells and the tumor against which an immune response is needed.

15

20.   The method of claim 18 or 19 wherein the dendritic-like cells of step (d) are produced by culturing precursors in the presence of cytokines.

21.   The method of any one of claims 18-20 wherein the tumor cells of step (c) are

20   drug-sensitive, said method further comprising, after step (e), killing unfused drug-sensitive immortal tumor cells by exposure to said drug.

22.   A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject a

25   plurality of dendritic-like cell/tumor cell hybrids.

23.   A method for producing an anti-tumor response in vitro, said method comprising co-cultivating in vitro a plurality of dendritic-like cell/tumor cell hybrids of claim 3 with immune cells from a mammalian subject in need of anti-tumor treatment.

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24.   A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject autologous immune cells activated in vitro by the method of claim 23.

5   25.   A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject a dendritic-like cell/tumor cell hybridoma.

26.   A method for producing an anti-tumor response in vitro, said method
10   comprising co-cultivating in vitro a dendritic-like cell/tumor cell hybridoma of claim 7 with immune cells from a mammalian subject in need of anti-tumor treatment.

27.   A method for producing an anti-tumor response in a mammalian subject in need of anti-tumor treatment, said method comprising administering to said subject
15   autologous immune cells activated in vitro by the method of claim 26.
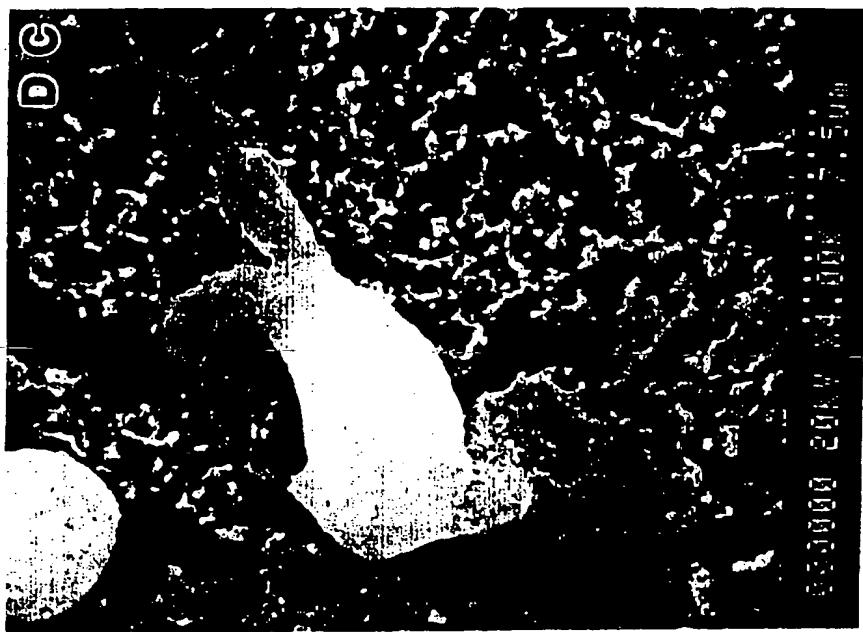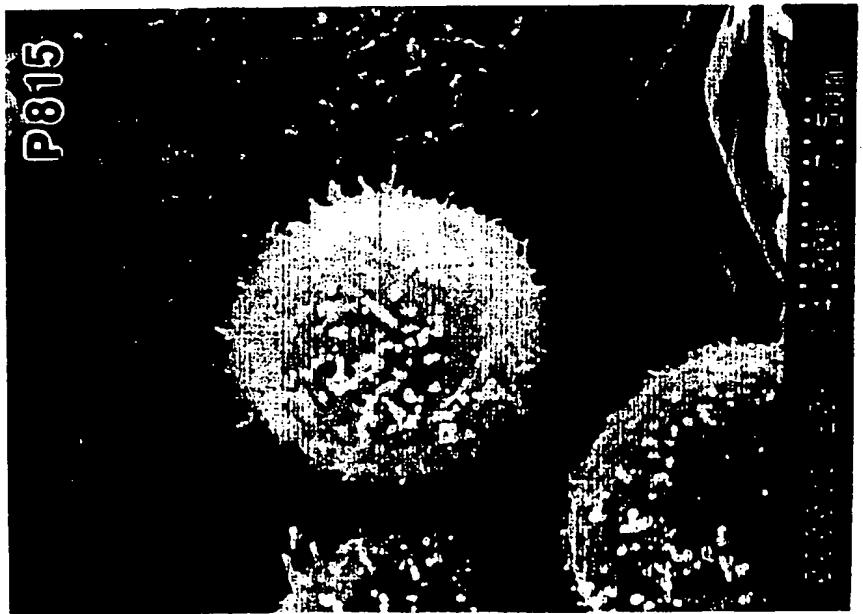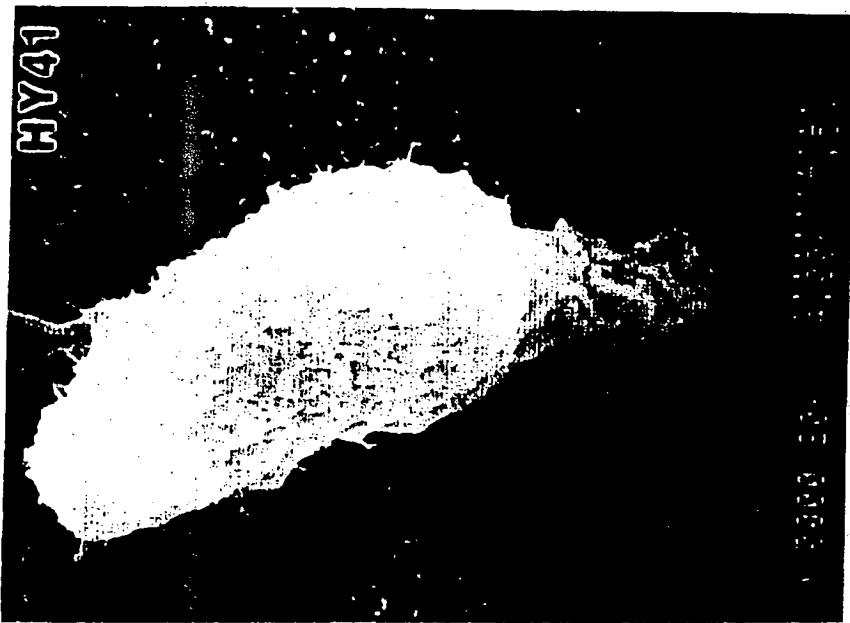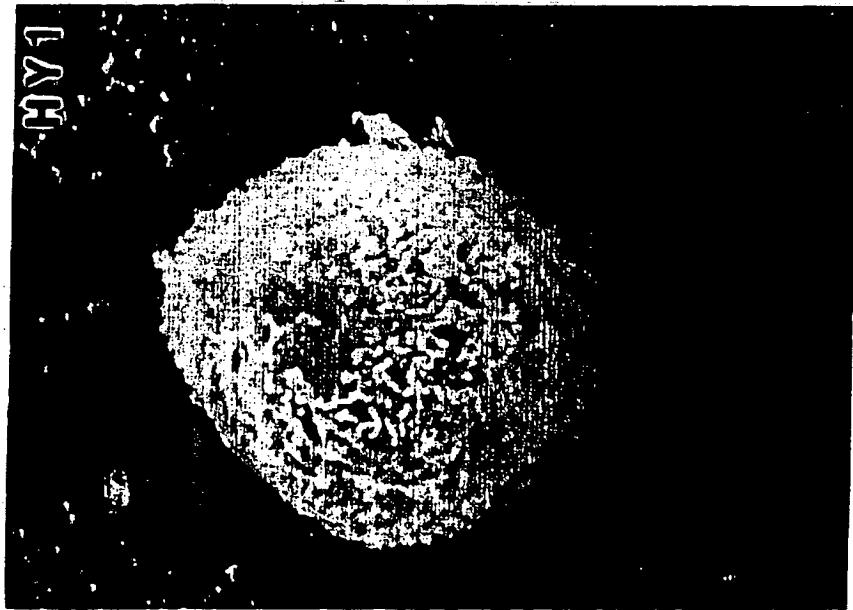
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FIG.1b

FIG.1a

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FIG.ld

FIG.lc

FIG. 2c

FIG. 2b

FIG. 2a

FIG.2e

FIG.2d

FIG. 3

FIG. 4

FIG. 5

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FIG. 6A

9/11
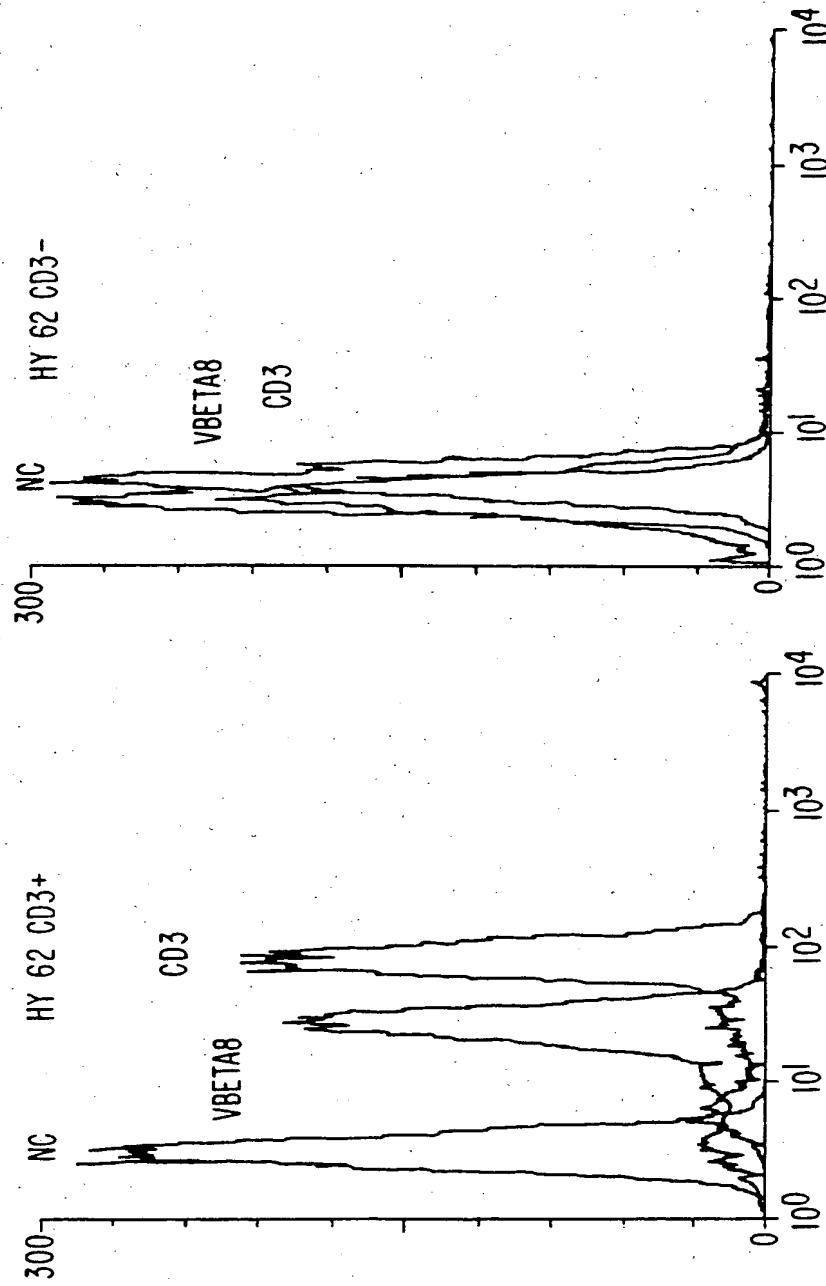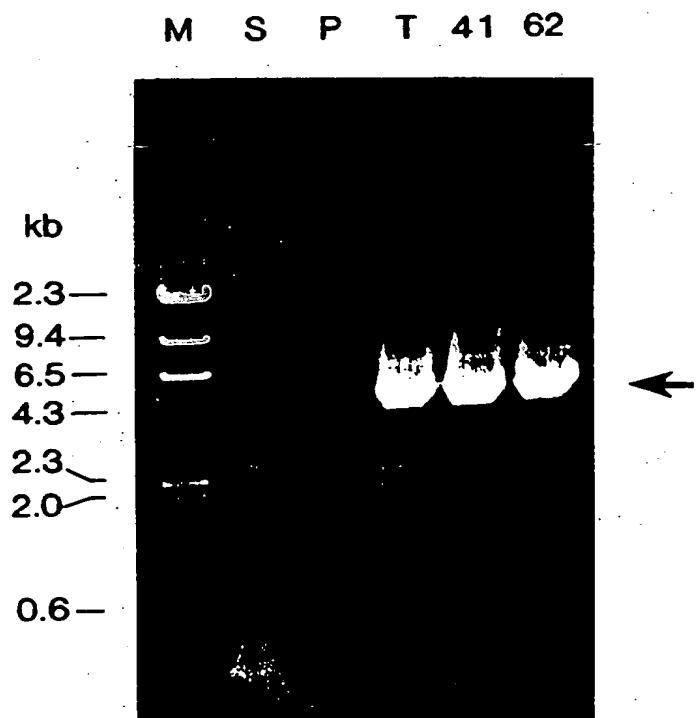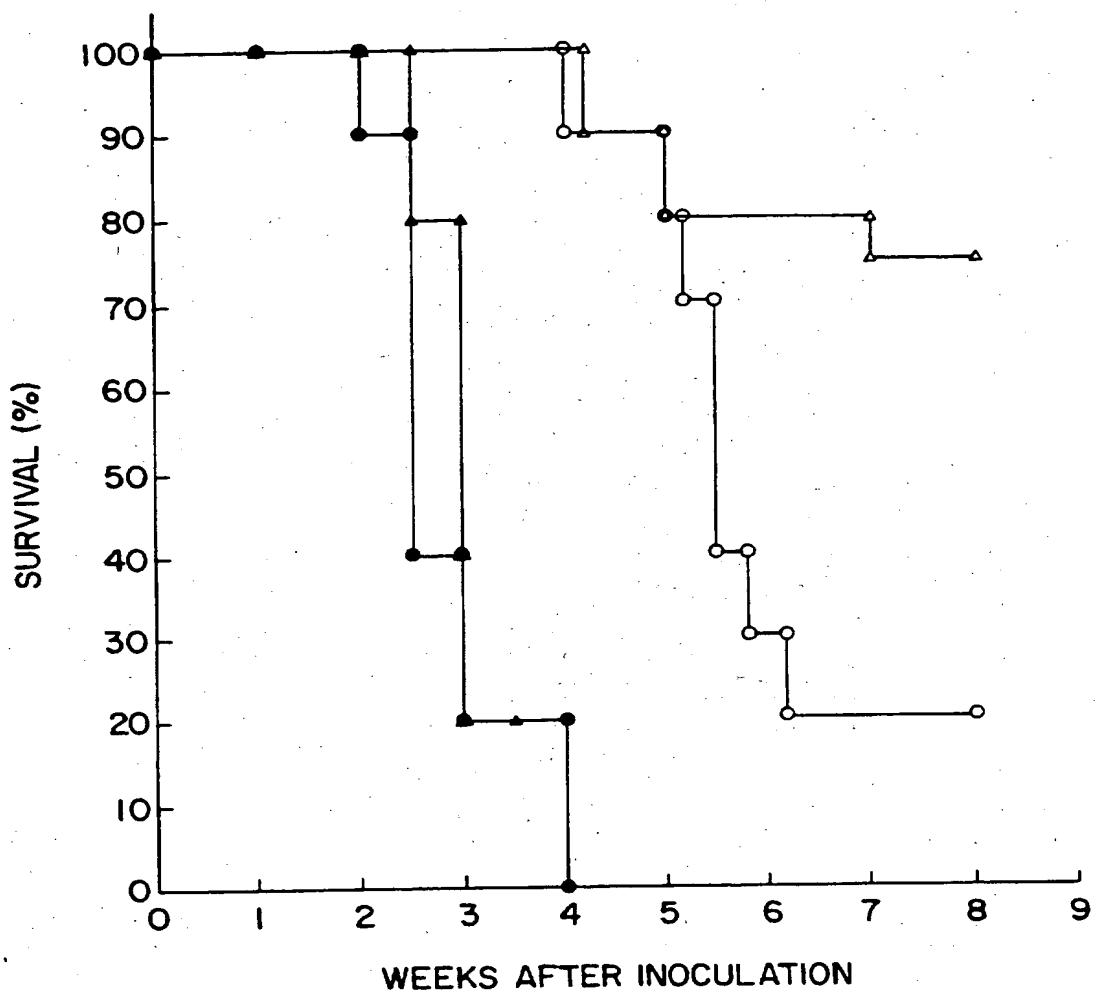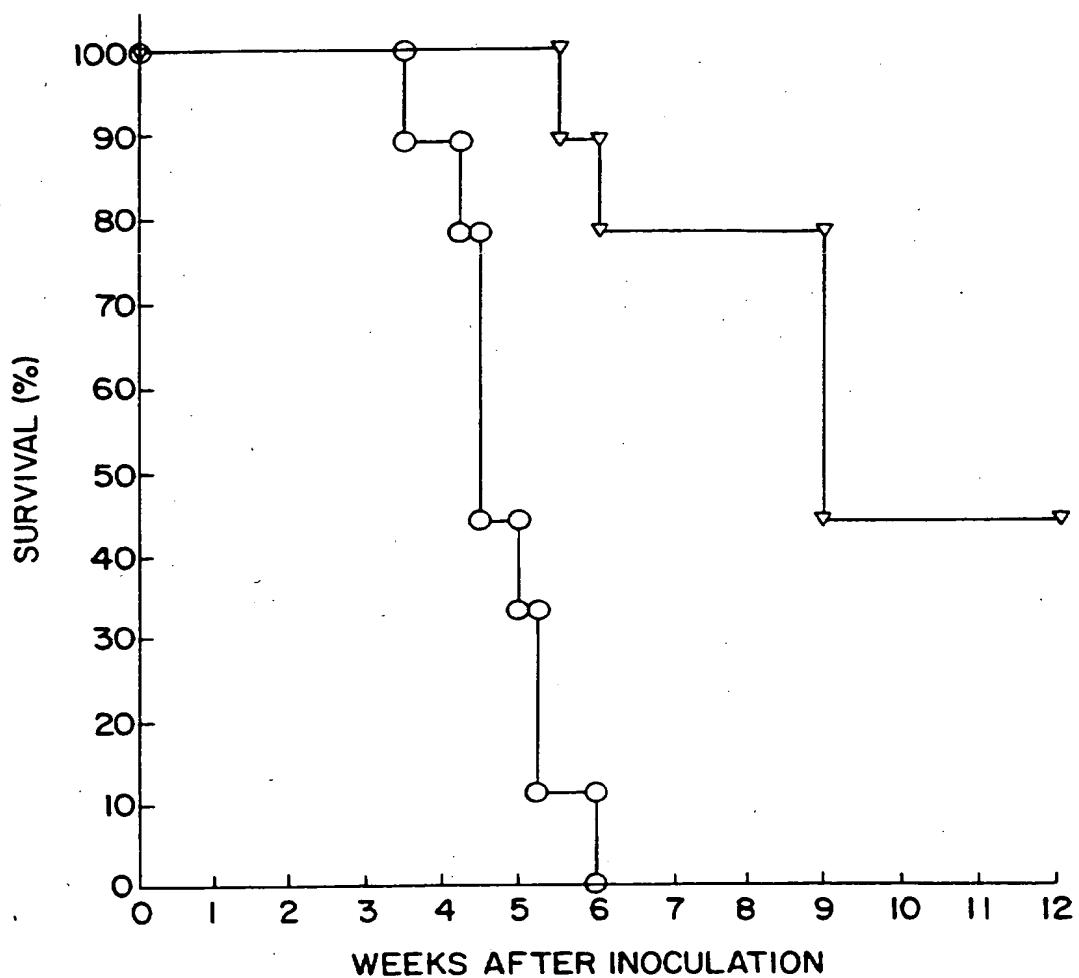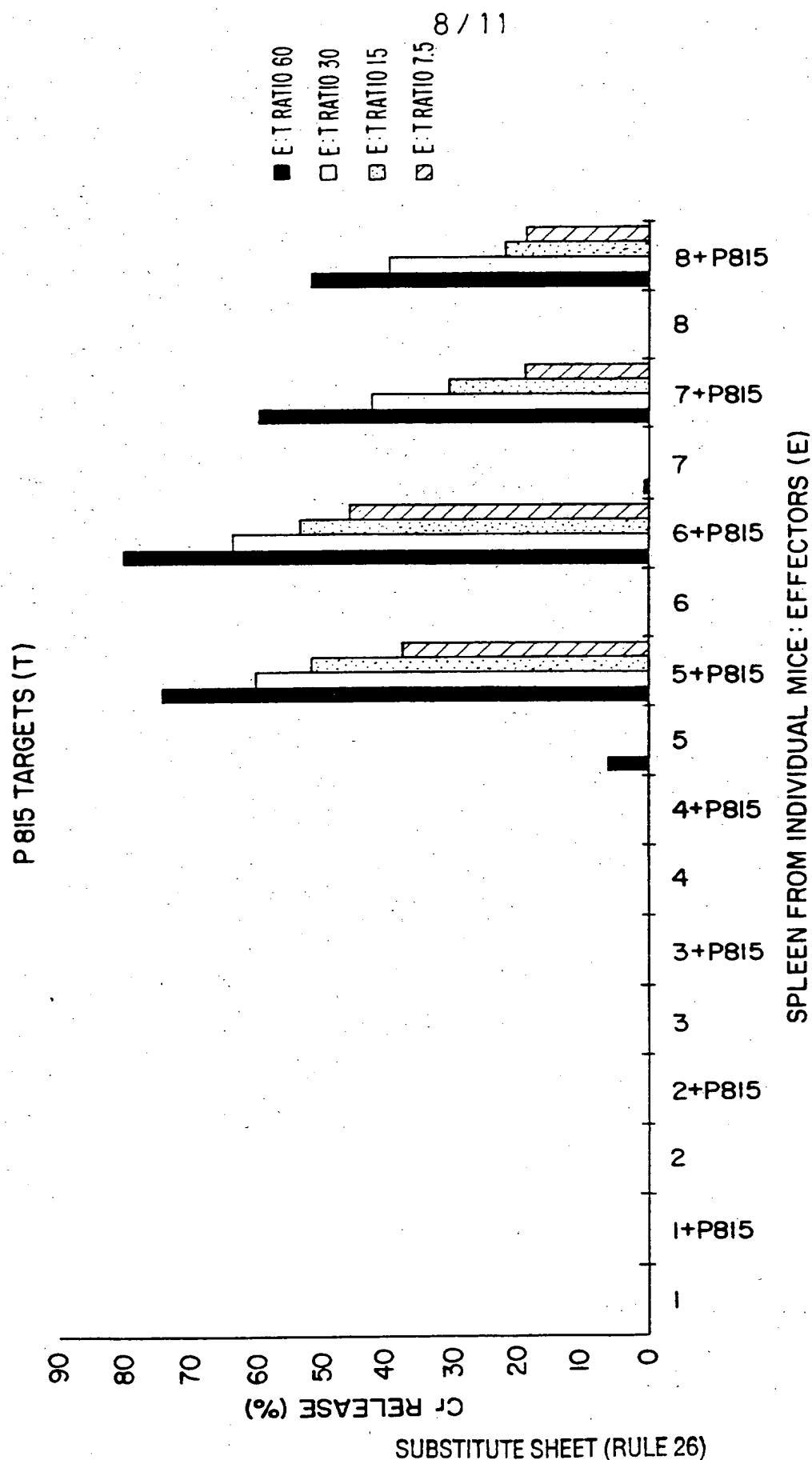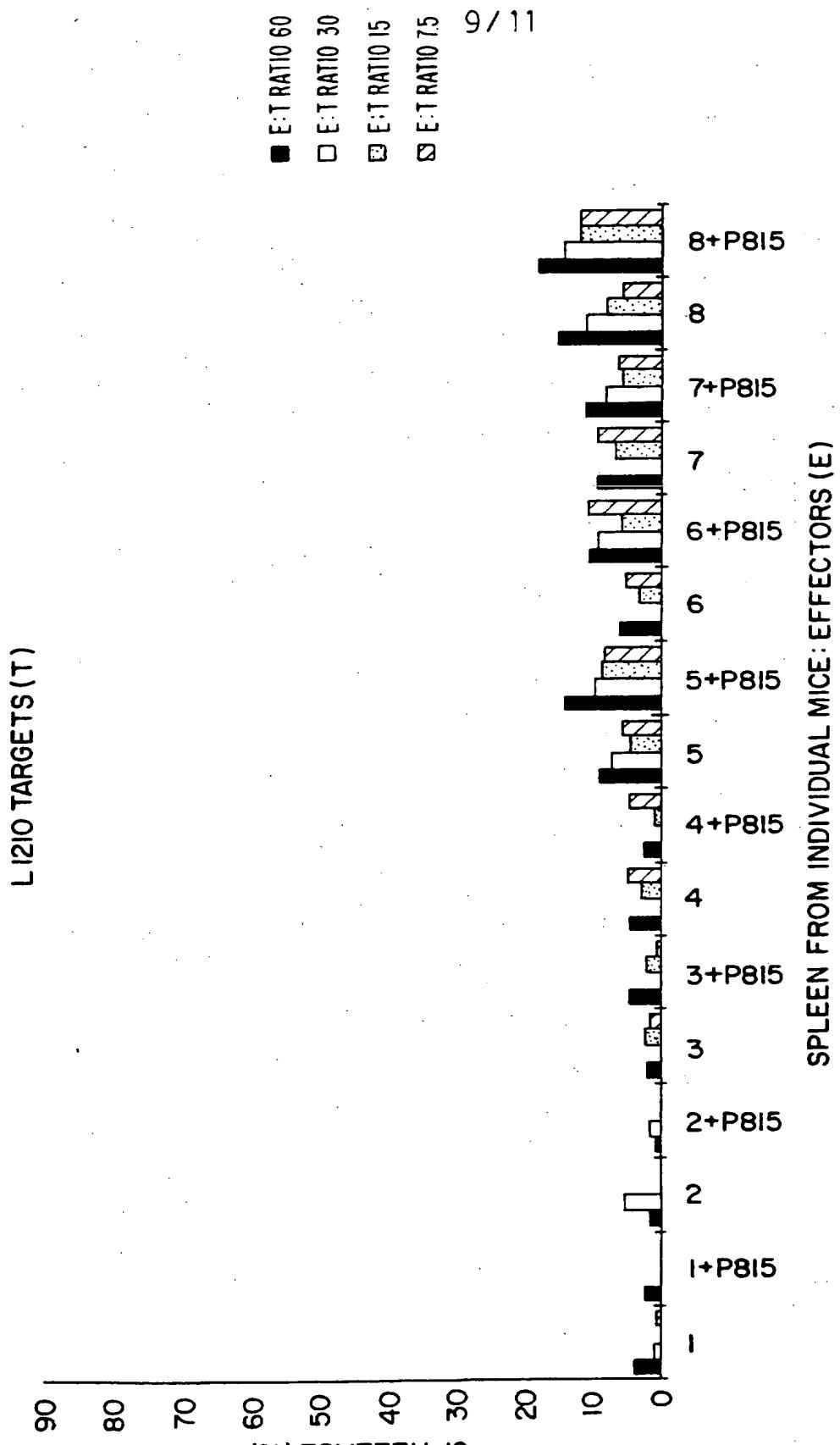
FIG. 6B

FIG. 7

FIG. 8a

FIG. 8b

| A. CLASSIFICATION OF SUBJECT MATTER |
| IPC(6) : A61K 35/14; C12N 5/22, 15/07 |
| US CL   : 435/172.2, 240.26; 424/93.7 |
| According to International Patent Classification (IPC) or to both national classification and IPC |

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

 U.S. : 435/172.2, 240.26; 424/93.7

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

 APS, CAS, Diolog, Medline, Biosis
 cell fusion/cell hybrids/dendritic cells/tumor antigens/antigen presentation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 263, Issued January 28 1994, Guo et al., "Effective Tumor Vaccine Generated by Fusion of Hepatoma Cells with Activated B Cells", pages 518-520, See entire document.	1-27
Y	Jornal of Eexperimental Medicine, Volume 178, Issued December 1993, Paglia et al., "Immortalized Dendriticl Cell Line Fully Competent in Antigen Presentation Intiates Primary T Cell Responses IN VIVO", Pages 1893-1901, See entire document.	1-27

| [X] Further documents are listed in the continuation of Box C. | [ ] See patent family annex. |

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be part of particular relevance	
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search	Date of mailing of the international search report
21 MAY 1996	14 JUN 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer NANCY A. JOHNSON
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Jornal Clinical Investigation, Volume 85, Issued March 1990, Markowitz et al., "Granulocyte-Macrophage Colony-Stimulating Factor Promotes Differentiation and Survival of Human Peripheral Blood Dendritic Cells IN VITRO", Pages 955-961, See entitre document.	1-27
Y	Journal of Experimental Medicine, Volume 172 Issued August 1990, Inaba et al., "Dendritic Cells Pulsed with Antigens in vitro can Prime Antigen-Specific, MHC-Restricted T Cells in situ", Pages 631-640, See entire document.	22, 25
Y	Proceedings National Academy of Sciences, Volum 85, Issued August 1988, Hauser et al., "Activation and Expansion of Hapten- and Protein-Specific T Helper Cells from Nonsensitized Mice", Pages 5625-5628, See entire document.	23, 24, 26, 27
P,Y	Jornal of Experimental Medicine, Volume 183, Issued JanuaryN 1996, Zitvogel et al., "Therapy of Murine Tumors with Peptide-Pulsed Dendritic Cells: Dependence on T Cells, B7 Costimulation, and T Helper Cell 1-Associated Cytokines", Pages 87-97, See entire document.	18
Y	The Journal of Immunology, Volume 146, Issued May 15 1991, Grabbe et al., "Tumor Antigen Presentation by Murine Epidermal Cells", Pages 3656-3661, See entire document.	18
A	Annual Review of Immunology, Volume 9, Issued 1991, Steinman, "The Dendritic Cell System and its Role in Immunogenicity", Pages 271-296, See entire document.	1-27
A	Journal Experimental Medicine, Volume 176, Issued December 1992, Inaba et al., "Generation of Large Numbers of Dendritic Cells from Mouse Bone Marrow Cultures Supplmented with Granulocyte Colony-Stimulating Factor", Pages 1693-1702, See entire document.	1-27
A	Jornal Experimental Medicine, Volume 175, Issued May 1992, Inaba et al., "Identification of Proliferating Dendritic Cell Precursors in Mouse Blood", Pages 1157-1167, See entire document.	1-27

FIG.1b

FIG.1a

FIG.ld

FIG.lc

FIG.2a   FIG.2b   FIG.2c

FIG. 2e

FIG. 2d

FIG.3

FIG. 4

FIG. 5

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FIG. 6A

FIG. 6B

FIG. 7

FIG. 8a

FIG. 8b